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TITLE: Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms

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13. ABSTRACT (Maximum 200 Words) The purpose of this application is training in nutritional and molecular epidemiology with the eventual goal of establishing an independent investigator. The hypothesis major hypothesis of the project is that high folate intake is associated with a decreased breast cancer risk particularly among those with MTHFR, MTR, and MTRR polymorphisms. The specific aims of this postdoctoral training proposal are 1) further methodological training in the analysis of gene-gene and gene-environment interactions by studying folate intake and folate metabolic gene polymorphisms (MTHFR, MTR, MTRR) using data collected in a population-based breast cancer case-control study (approximately 3000 subjects), 2) training in the methodology of cohort studies through designing and implementing a newly proposed nested case-control study of breast cancer (350 pairs) to examine folate intake, plasma folate, and metabolic gene polymorphisms, 3) coursework in nutrition and cancer biology and 4) participation in the field work of a recently submitted breast cancer case-control study and 5) development of a grant proposal examining folate, global DNA methylation and uracil misincorporation in breast cancer risk.				
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Folate and Breast Cancer: Role of Intake, Blood Levels, and Metabolic Gene Polymorphisms.

INTRODUCTION

Folate, a B vitamin found naturally in many food sources particularly in dark green leafy vegetables, is essential for regenerating methionine, the methyl donor for DNA methylation, and for producing the purines and pyrimidine thymidylate required for DNA synthesis and repair. Evidence for its potential role in carcinogenesis is encouraging. Several genes involved in the metabolism of folate have known polymorphisms and the combined effect of these polymorphisms with folate intake may affect breast cancer risk. *MTHFR* irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group for the de novo methionine synthesis and DNA methylation. Two common polymorphisms in the *MTHFR* gene have been identified both of which result in decreased *MTHFR* activity (C677T, A1298C). Methionine synthase, a vitamin B12-dependent enzyme that converts homocysteine to methionine by the transfer of a methyl group from 5-methyltetrahydrofolate, is encoded by the *MTR* gene and a polymorphism has been identified (A2756G). Methionine synthase reductase reductively activates methionine synthase from its inert to reactive form. The gene, *MTRR*, has been identified along with a polymorphism (A66G). *MTHFR* and *MTR* polymorphisms have been associated with reduced colorectal cancer risk. A small hospital-based case-control study found an increased risk with *MTHFR* 677T and no association with *MTR*, results not consistent with the more extensive colorectal cancer results. No breast cancer study has evaluated the role of *MTRR*. **Purpose:** The specific aims of this postdoctoral training proposal are 1) training in the analysis of gene-gene and gene-environment interactions by studying folate intake and folate metabolic gene polymorphisms (*MTHFR*, *MTR*, *MTRR*) in a population-based breast cancer case-control study (approximately 3000 subjects), 2) training in the methodology of cohort studies through a newly proposed nested case-control study of breast cancer (350 pairs) examining folate intake, plasma folate, and metabolic gene polymorphisms, 3) coursework in nutrition and cancer biology and 4) development of a grant proposal examining folate and global DNA methylation in breast cancer risk. **Scope:** Most established risk factors for breast cancer are very difficult to modify, therefore, identifying modifiable factors is essential to prevent the disease globally. The US and Canada currently fortify all cereal grain foods with folic acid, although most other countries do not. A serendipitous result may be an eventual decrease in breast cancers. It is necessary, therefore, to assess the relationship between folate and breast cancer so that high-risk groups may be targeted and international breast cancer incidence decrease.

BODY

Approved Statement of Work

Task 1. Undergo course training in nutrition and molecular biology, Months 1-22:

- a. Take 1 course in the Vanderbilt Department of Molecular Biology (Fall Semester, 2002), Introduction to Cell Biology: Months 6-10.
- b. Take 1 course in the Vanderbilt School of Medicine (Spring Semester 2003), Introduction to Clinical Nutrition: Months 11-12.
- c. Take 1 course in the Vanderbilt Department of Molecular Biology (Spring Semester 2003), Cancer Biology: Months 11-15.
- d. Take 1 course in the Vanderbilt Department of Biochemistry (Fall Semester, 2003), Molecular Aspects of Cancer Research: Months 18-22.

1a. Introduction to Cell Biology will be completed in Fall Semester 2003.

1b. Introduction to Clinical Nutrition will be replaced with an independent study of nutrition.

1c. Cancer Biology was completed in December 2001.

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Task 2. Undergo training in the analysis of gene-gene and gene-environment interactions and the associations of folate intake and folate metabolizing gene polymorphisms using data from a population-based case-control study of 3000 subjects in Shanghai: Months 1-24

- a. Analyze the association between MTHFR polymorphisms and breast cancer risk and prepare a manuscript to report the findings: Months 1-10.
- b. Analyze and publish the joint effect of MTHFR, MTR, and MTRR polymorphisms, folate intake, and breast cancer risk: Months 10-18.

2a. The manuscript was completed and submitted to Carcinogenesis for publication. A copy of this manuscript is included in Appendix 1. We found that MTHFR genotypes were not associated with breast cancer risk, however, MTHFR C677T genotypes appeared to modify the association between folate and breast cancer risk.

Task 3. Undergo training in the methodology of cohort studies and to evaluate the association of folate with breast cancer risk using data from a prospective cohort study of 75, 000 Chinese women in Shanghai: Months 1-36.

- a. Design a nested case-control study (350 matched pairs) within the Shanghai Women's Health Study for the prospective evaluation of folate intake, plasma folate, and metabolic gene polymorphisms in relation to breast cancer risk: Months 1-19.
- b. Prepare blood samples for relevant assays: Months 1-19.
- c. Analyze and publish the relationship between folate intake (all 75, 000 women), plasma folate (700 subjects in a nested case-control study) and breast cancer risk. Months 21-26.
- d. Analyze and publish the relationship between metabolic gene polymorphisms and breast cancer risk: Months 26-36.
- e. Analyze the joint effect of metabolic gene polymorphisms, plasma and dietary folate, and breast cancer risk: Months 26-36.

3a and b. Follow-up of all participants is on-going. It is expected that more than 200 breast cancer cases with blood samples will occur by the end of 2003. At that time, the nested-case control study will be designed and all blood samples will be assayed. Other cases and controls will be accrued prospectively from that point.

Task 4. Undergo training in implementation and administration of breast cancer epidemiological studies by participating in the field work of the Nashville Breast Health Study, a new case-control study. Months 1-36.

- a. Assist in the development of study instruments, materials, and procedures: Months 1-6.
- b. Participate in subject identification and recruitment: Months 3-36.
- c. Prepare manuscripts for publication: Months 26-36.

4a and b. I have developed and modified the following study instruments: telephone questionnaire, call logs, and other procedural forms. I have developed protocols for patient recruitment, random digit dialing, interviewer training and other interviewer procedures. I have designed a database for patient tracking and data entry and several reports to monitor study progress.

Task 5: Prepare a grant proposal for continuation. Months 28-34.

- a. Develop and submit a grant proposal to expand the sample size of the nested case-control study to evaluate folate, global DNA methylation, and uracil misincorporation in lymphocytes in relation to breast cancer risk.

5. Ongoing.

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KEY RESEARCH ACCOMPLISHMENTS

- **December 2001:** Attended Cancer Biology in the Vanderbilt Department of Molecular Biology.
- **Summer 2002:** Contributed to the Nashville Breast Health Study grant submission.
- **November 2002:** Prepared the manuscript "*MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study" for submission to *Carcinogenesis*. The manuscript is included as Appendix 1.
- **January 2003:** Presented the poster "*MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study" at the American Association for Cancer Research Molecular and Genetic Epidemiology of Cancer Conference.
- **May 2002-ongoing:** Project management of the Nashville Breast Health Study

REPORTABLE OUTCOMES

1. The manuscript "*MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study" has been prepared and submitted. It is included in Appendix 1.
2. The poster "*MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study" was presented at the AACR Molecular Epidemiology of Cancer Conference.
3. I was accepted to the American Association for Cancer Research Pathobiology of Cancer Workshop.

CONCLUSIONS

Results from the *MTHFR* and breast cancer risk manuscript indicate that *MTHFR* genotype alone is not associated with breast cancer risk. However, *MTHFR* genotype may affect the degree to which folate is protective in breast cancer risk. This is one of the first and largest studies to examine this association. It will be important to verify this relationship in the nested case-control study supported by this grant.

The experience gained from participating in the Nashville Breast Health Study has allowed me to become involved in the study design and management of two additional cancer epidemiology studies. This will further expand my ability to become an independent investigator.

REFERENCES

1. Martha J. Shrubsole, Yu-Tang Gao, Qiuyin Cai, Xiao Ou Shu, Qi Dai, James R. Hébert, Fan Jin, and Wei Zheng. *MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. 2003. Submitted.
2. Martha J. Shrubsole, Yu-Tang Gao, Qiuyin Cai, Xiao Ou Shu, Qi Dai, James R. Hébert, Fan Jin, and Wei Zheng. *MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. 2003. Presented at the AACR Molecular and Genetic Epidemiology of Cancer.

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Appendix 1

Martha J. Shrubsole, Yu-Tang Gao, Qiuyin Cai, Xiao Ou Shu, Qi Dai, James R. Hébert, Fan Jin, and Wei Zheng. *MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study. 2003. Submitted.

***MTHFR* polymorphisms, dietary folate intake, and breast cancer risk: Results from the Shanghai Breast Cancer Study¹**

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Running title: *MTHFR* polymorphisms and breast cancer risk

Key words: breast cancer, folate, epidemiology, *MTHFR*, etiology

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ABSTRACT

Folate plays an important role in DNA methylation, synthesis, and repair; dietary intake of this nutrient has been associated with breast cancer. The folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR) is polymorphic at nucleotides 677 (C→T) and 1298 (A→C) resulting in allozymes with decreased activity. We evaluated these two common polymorphisms and their modifying effects on the association of folate intake with breast cancer risk in a population-based case-control study of 1459 breast cancer cases and 1556 controls conducted in urban Shanghai during 1996-1998. All subjects were interviewed in person using a comprehensive questionnaire, including a food frequency questionnaire. *MTHFR* genotypes were evaluated in 1144 cases and 1236 controls using a polymerase chain reaction restriction fragment-length polymorphism-based assay. Unconditional logistic regression models were used to calculate odds ratios (OR) and their 95% confidence intervals (95% CI), after adjusting for potential confounding factors. Cases and controls were similar in the distribution of *MTHFR* polymorphisms at codons 677 (41.4% of cases and 41.8% of controls carried the T allele) and 1298 (17.6% of cases and 17.5% of controls carried the C allele). An inverse association of breast cancer risk with folate intake was observed in all genotype groups. The association, however, was substantially stronger in subjects with the 677TT genotype (OR=4.75, 95% CI=1.82-12.4 for the lowest vs. highest folate intake) than those with either the CC (OR=2.51, 95% CI=1.29-4.89 for the lowest vs. highest folate intake) or CT genotypes (OR=1.65, 95% CI=0.99-2.75 for the lowest vs. highest folate intake) (*p* for interaction, 0.05). No modifying effect of the A1298C genotypes on the association of folate intake with breast cancer risk was observed. Results of this study suggest that the *MTHFR* C677T polymorphisms may modify the association between dietary folate intake and breast cancer risk.

Introduction

Folate is involved in DNA methylation, synthesis, and repair. Low intake of folate may increase risk for several cancers, including breast cancer (1;2). The enzyme methylenetetrahydrofolate reductase (MTHFR) irreversibly catalyzes 5,10-methylenetetrahydrofolate (THF) to 5-methyl THF, the donor for the remethylation of homocysteine to methionine, the precursor for the universal methyl donor, *S*-adenosylmethionine (3;4). Folate that is not converted through this pathway can be used for purine synthesis or the conversion of uracil to thymine, which is used for DNA synthesis and repair (5).

Two common polymorphisms in the *MTHFR* gene have been characterized (6;7). The 677C → T polymorphism codes for an alanine to valine substitution in the N-terminal catalytic domain and results in an allozyme with approximately 30% of the activity of the wild-type protein (6). The A → C polymorphism at nucleotide 1298 codes for an alanine to glutamine substitution in the C-terminal regulatory domain (7). Individuals homozygous for the 1298C allele have approximately the same enzyme activity as those heterozygous for the 677T allele (7).

The C677T polymorphism has been examined in relation to several cancers (2;8). In most studies of colorectal neoplasms, the *MTHFR* 677TT genotype has been associated with an overall reduction in risk, reduced risk among those with higher intakes of folate (9-12), or increased risk among those with lower folate intakes (12-14). *MTHFR* has not been as well studied in relation to breast cancer risk (15-19). Only five small studies have evaluated the association between *MTHFR* genotype and breast cancer. The results from these studies have been inconsistent. Only one study assessed both the C677T and A1298C polymorphisms and their possible joint effect with folate intake (17). However, in that study, only 60 cases were included. We reported recently that folate intake was inversely associated with breast cancer risk in a large population-

based case-control study among Chinese women in Shanghai (20). In an extension of these results, we investigated whether this association may be modified by *MTHFR* genotypes.

Materials and methods

The Shanghai Breast Cancer Study is a population-based case-control study conducted in urban Shanghai, China during 1996-1998. This study was approved by the committees for the use of human subjects in all collaborating institutions. Detailed study methods have been previously published(21).

Subjects: All incident breast cancer cases newly diagnosed during the study period and meeting the eligibility criteria were identified through a rapid case-ascertainment system supplemented by the Shanghai Cancer Registry and were approached for participation in the study. Eligibility criteria for the study were as follows: 25-64 years of age, resident of urban Shanghai, no previous history of any cancer, and alive at the time of interview. In all, 1,602 eligible cases were identified, of whom, 1,459 (91.1%) completed in-person interviews. The median interval from cancer diagnosis to the in-person interview was 64 days. With the exception of a breast cancer diagnosis, controls had inclusion criteria identical to those of the cases and were frequency matched on age (5 years intervals) to the expected age distribution of the cases. In all, 1,724 eligible controls were randomly selected from the Shanghai Resident Registry. Of these, 1,556 (90.3%) completed in-person interviews.

Data and Biologic Sample Collection: All subjects completed an in-person interview that used a structured questionnaire and incorporated anthropometric measurements. Dietary intakes were assessed using a 76-item food frequency questionnaire (FFQ) that captured over 80% of food intake in this population. Each subject was asked about the frequency with which she ate a

specific food (daily, weekly, monthly, yearly, or never), followed by a question on the amount typically eaten. Dietary intakes of total folate and folate cofactors were derived from the FFQ (20). Blood samples were collected from 1193 (82%) cases and 1310 (84%) controls and used in this study for genotyping assays.

Laboratory methods: Genomic DNA was extracted from blood samples with the Puregene® DNA isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol. Genotyping for the *MTHFR* C677T and A1298C polymorphisms were performed using PCR-RFLP methods reported by Frosst et al. (6) and Weisberg et al. (7) with minor modifications. The primers for C677T analysis were: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' (exonic) and 5'-AGGACGGTGCGGTGAGAGTG-3' (intronic). The primers for A1298C analysis were: 5'-GGGAGGAGCTGACCAGTGCAG-3' and 5'-GGGGTCAGGCCAGGGGCAG-3'. The PCR reactions were performed in a Biometra® TGradient Thermocycler. Each 20 µl of PCR mixture contained 10 ng DNA, 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0), 1.5 mM MgCl₂, 0.2 mM each of dNTP, 0.5 mM of each primer, and 1 unit of Taq DNA polymerase. The reaction mixture was initially denatured at 94°C for 3 min. For C677T polymorphisms, PCR was performed in 30 cycles of 94°C for 45 sec, 65°C for 45 sec, and 72°C for 45 sec. For A1298C polymorphisms, PCR was performed in 35 cycles of 94°C for 45 sec, 65°C for 45 sec, and 72°C for 45 sec. The PCR was completed by a final extension cycle at 72°C for 7 min.

For C677T polymorphisms, each PCR product (10 µl) was digested with 10 units of *Hinf*I at 37°C for 3 hours. The DNA fragments were then separated using 3% agarose gel and detected by ethidium bromide staining. The C→T substitution at nucleotide 667 creates a *Hinf*I digestion site. The PCR product (198 bp) with T allele was digested to 2 fragments (175 bp and 23 bp), whereas the PCR product with wild type C allele cannot be cut by *Hinf*I. For A1298C

polymorphisms, each PCR product (10 µl) was digested with 5 units of *Fnu4HI* at 37°C for 3 hours followed by 3% agarose gel electrophoresis and ethidium bromide staining. The A→C substitution at nucleotide 1298 creates a *Fnu4HI* site. The PCR product (138 bp) with C allele was digested to 2 fragments (119 bp and 19 bp), whereas the PCR product with wild type A allele can not be cut by *Fnu4HI*.

Quality control (QC) samples were included in various batches of samples assayed for the polymorphisms. The consistency rate was 98.5% in 119 QC samples that were repeated in the genotyping assays with their identities unknown to lab staff. Excluding a few subjects for whom sufficient DNA was not available or for whom the genotyping assay failed, genotyping data were obtained from 1112 cases and 1160 controls for C677T and 1121 cases and 1208 controls for A1298C polymorphisms. Because few women in the study consumed alcohol, a factor that may increase folate requirements, and because the data on the folate content of vitamins were not available, all analyses involving folate or its cofactors were limited to the cases (92.0%) and controls (91.1%) who were known not to consume alcohol regularly and not to take vitamin supplements.

Data Analysis: Odds ratios were used to measure the association of breast cancer risk with *MTHFR* genotype. Unconditional logistic regression models were used to obtain maximum likelihood estimates of the odds ratios and their 95% confidence intervals, after adjusting for potential confounding variables. Risk factors previously identified as having an independent association with breast cancer were controlled in all models. These included age, personal history of fibroadenoma, age at first live birth, physical activity, waist-to-hip ratio, and daily meat intake. Age was included as a continuous variable throughout, and categorical variables were

treated as indicator variables in the model. Quartile and tertile distributions of dietary intakes among controls were used to categorize all dietary intake variables. In the analyses including dietary factors, energy adjustment was performed using the standard multivariate method (22). Tests for trend were performed by entering categorical variables as continuous. Stratified analyses were used to evaluate the potential modifying effect of age, menopausal status, and folate and folate cofactor intakes on breast cancer risk associated with *MTHFR* genotypes and of *MTHFR* genotypes on breast cancer risk associated with folate intake. Tests for multiplicative interaction were done by including multiplicative variables in the logistic model and performing the likelihood ratio test. All statistical tests were based on two-sided probabilities using SAS, version 8.2 (SAS Institute, Inc., Cary, NC).

Results

In Table I, comparisons between cases and controls on select demographic factors, established risk factors, and dietary factors are presented. Cases were, in general, more highly educated, more likely to have a history of fibroadenoma, younger at menarche, older at first live birth and menopause, less likely to be physically active, and more likely to have a higher BMI and WHR than controls. Cases also had higher average daily intakes of animal foods, methionine, and vitamin B6 and lower average daily intake of folate than controls.

-Table I near here-

The frequencies of *MTHFR* alleles and genotypes by case-control status and the association between *MTHFR* genotypes and breast cancer risk are presented in Table II. The frequencies of

the 677T and 1298C alleles were 0.41 and 0.18, respectively among the controls. These were virtually identical to the frequency among the cases. Among the controls, the distributions of the *MTHFR* genotypes did not differ from the predicted distribution under Hardy-Weinberg equilibrium ($p=0.44$ for the C677T polymorphisms and $p=0.58$ for the A1298C polymorphisms). Risk of breast cancer did not differ statistically for the C677T or A1298C genotypes or for their combination.

-Table II near here-

To evaluate the potential modifying effect of age and menopausal status with *MTHFR* genotype, stratified analyses were performed (Table III). Regardless of stratum, risk of breast cancer did not statistically significantly vary by C677T or A1298C genotypes. To assess whether an association with a particular genotype was confounded by the other polymorphism, these stratified analyses were also performed for the joint association of the *MTHFR* genotypes. There was still no apparent association between risk of breast cancer and genotype, regardless of age or menopausal status stratum.

-Table III near here-

The association of folate with breast cancer risk is presented in Table IV stratified by *MTHFR* genotype. When stratified by C677T genotype, low intake of folate was associated with an increased risk of breast cancer among all genotypes, particularly subjects with the TT genotype (OR=4.75; 95% CI: 1.82-12.4 for lowest vs. highest quartile). There was a significant

multiplicative interaction between folate intake and C677T polymorphism in relation to breast cancer risk ($p=0.05$). When stratified by the A1298C polymorphism, elevated ORs were observed to be associated with folate intake in both AA or AC/CC groups, although the trend was statistically significant only in the AA group. To examine further the C677T association, analyses were restricted to 1298 AA individuals because of a small sample size for the AC and CC genotypes. Again, low intake of folate was associated with increased risk for all groups stratified by the C677T genotypes, and the increased risk was greatest among those with 677TT genotype (OR=5.31, 95% CI: 1.99-14.2, p for trend=0.002, p for interaction=0.06). Analyses also were performed to calculate odds ratios for *MTHFR* genotypes when stratified by folate intake. The OR associated with the 677TT genotype was reduced among subjects with high folate intake (OR=0.73, 95% CI: 0.41-1.31 for TT vs. CC) and elevated among subjects with low folate intake (OR=1.33, 95% CI: 0.78-2.28 for TT vs. CC) although both ORs were not statistically significantly different from 1.0.

-Table IV near here-

In Table V, the associations of folate intake with breast cancer risk stratified by folate cofactor intake and *MTHFR* C677T genotype are presented. With the exception of the high vitamin B6 stratum, low folate intake was associated with an elevated risk of breast cancer, particularly among subjects with the TT genotypes. The modifying effect of the C677T genotype appears to be stronger among subjects with a high or intermediate intake of vitamin B12 or methionine than those with a low intake of either of these two folate cofactors. For high intake of vitamin B6, however, low intake of folate was associated with a decreased risk of breast cancer among those

with the CT or TT genotypes. None of the tests for multiplicative interactions, however, was statistically significant.

-Table V near here-

Discussion

We found in this case-control study that there was no statistically significant association between the risk of breast cancer and *MTHFR* C677T or A1298C genotypes. However, *MTHFR* C677T genotype was a statistically significant effect modifier of the association between folate intake and breast cancer risk. Among those with the 677TT genotype, low folate intake was associated with a more substantial increased risk than those with other genotypes. These findings are new and consistent with the possible role of *MTHFR* and folate in the etiology of cancer.

MTHFR polymorphisms have not been adequately investigated in relation to breast cancer risk. Only five previous small studies have examined *MTHFR* polymorphisms and breast cancer risk (15-19). In the first, a study among Jewish women, *MTHFR* C677T genotype was determined in 491 women with sporadic (n=355) or hereditary (n=136) breast and/or ovarian cancer and in 69 asymptomatic BRCA1/2 mutation carriers. The prevalence of the T allele was not significantly different between sporadic cases and the asymptomatic carriers, women diagnosed at a young and older age, and BRCA1/2 carriers with and without cancer. The prevalence of the T allele was more frequent among women with bilateral breast cancer or with both breast and ovarian cancers than among women with only unilateral breast cancer. In the second study, a hospital-based case-control study among post-menopausal Caucasian women (149 cases and 171 controls), it was reported, however, that the *MTHFR* 677T (val) allele was

more prevalent in cases than controls (15), which is in contrast to the results from the third case-control study conducted in the UK (62 cases, 66 controls), the only previous study that reported risk of breast cancer associated with both the C677T and A1298C polymorphisms or evaluated the relationship with folate (17). This study reported breast cancer risk was reduced among those homozygous for the 677T allele (OR=0.39; 95% CI: 0.12-1.24) or 1298C allele (OR=0.24; 95% CI: 0.06-0.97). However, no modifying effect of the *MTHFR* C677T genotype was noted on the association between folate intake and breast cancer risk. There was some evidence of a joint association of folate and the A1298C genotype but the sample size was not large enough to examine this association. In a UK study of women with early age of onset or bilateral or family history of breast cancer and a mixture of hospital and staff volunteer controls, the presence of at least one T allele was associated with an increased risk among those diagnosed before 40 but not among those with a history of familial or bilateral breast cancer (18). In the final study, a small US study of breast cancer cases and benign breast disease controls, the presence of a T allele was associated with an increased risk of premenopausal breast cancer but not with postmenopausal breast cancer.

We did not find an overall reduced risk of breast cancer associated with *MTHFR* 677TT or 1298CC genotypes, which is not consistent with the British study of breast cancer¹⁷ and some of the previous studies for other cancers. Two of three case-control studies of colorectal cancer and the *MTHFR* C677T polymorphism observed an overall reduction in risk associated with the TT genotype (9;10) as did studies of oral cancer (23) and adult acute lymphocytic leukemia (24). However, other studies of colorectal cancer (11), colorectal adenoma (12;13;25), gastric cancer (8), lung cancer (26), and acute myeloid leukemia (24) found no association or an increased risk

of cancer for individuals with the TT genotype. Our observation for a stronger inverse association of folate intake and breast cancer risk among women with the TT genotype is supported by the majority of studies examining a similar association for other cancers (9-11;13;14;23), and is consistent with the role of folate in breast carcinogenesis. We, and others, have previously found a decreased risk of breast cancer among those with high intake level of folate (20;27-33). Low folate intake is associated with increased misincorporation of uracil and chromosome breaks (34;35) and aberrant DNA methylation (35;36). The critical factor in breast carcinogenesis may be an appropriate balance between the availability of SAM for DNA methylation and 5,10-methylene THF for DNA synthesis. It is plausible that individuals with the 677TT genotype are particularly susceptible to the carcinogenic consequences of folate insufficiency. This genotype, in the presence of low folate, is associated with higher levels of homocysteine, lower levels of methylated folates and, therefore, reductions in genomic DNA methylation (37;38). Our finding for a positive association of C677T genotype (OR=1.33, 95% CI: 0.78-2.28) appears to support this notion. Conversely, in folate-replete conditions, the availability of 5,10-methylene THF for nucleotide synthesis may be adequate or increased for these individuals due to the genetically-determined decreased activity of MTHFR. This could explain the lower risk of this genotype among those with high folate levels in this (OR=0.73, 95% CI: 0.41-1.31 for 677TT and OR=0.58, 95% CI: 0.30-1.12 for 677TT/1298AA) and other studies (9;11;13;14). Therefore, the effect of *MTHFR* on breast cancer risk in a particular population may depend on the intake level of folate in that population. With increased folic acid fortification in the United States population, the general intake of folate may be higher than that from the Chinese whose folate intake is primarily obtained unfortified diets. This may explain, in part, the overall absence of association of *MTHFR* genotype with breast cancer risk in our study.

The relationship between folate metabolism and carcinogenesis is likely to be a complex biological sum of genetic and nutritional differences. In our study, the association of folate and breast cancer risk was similar for all genotypes when intakes of vitamin B12, B6 or methionine were low. Vitamin B12, vitamin B6, and methionine all have important roles in one-carbon metabolism; vitamin B12 is a cofactor for the transfer of the methyl group from folate to methionine, vitamin B6 is a coenzyme for the formation of 5,10-methylene THF and the catabolism of homocysteine, and methionine is the precursor for SAM. It is possible that below a certain intake threshold of vitamin B12 and methionine the effect of *MTHFR* C677T genotype or folate intake is reduced or negated and that once this threshold is surpassed both folate and *MTHFR* genotype have a greater impact on breast cancer risk. The inverse association with breast cancer risk among those with a high vitamin B6 intake was unexpected and cannot be readily explained by the above rationale. This finding needs to be re-evaluated in future studies.

As with any case-control study, the potential for selection and recall biases must be considered. However, selection bias is unlikely to be a major issue in this study; both cases (91%) and controls (90%) had very high participation rates. Not only did this study have a high participation rate, it also had a high blood collection rate (over 80%). Although possible that cases and controls may have differentially recalled intakes of foods that contributed to the nutrients in this study, fruit and vegetable intake, the major contributors to folate, methionine, and vitamin B6 intakes, did not significantly differ between cases and controls. About half of cases were interviewed within 15 days (50%) of diagnosis and the majority were interviewed within 4 months (80%), thus, reducing potential recall bias attributable to dietary change related

to a diagnosis of cancer. In addition, recall of diet would unlikely be related to *MTHFR* genotype, and, therefore, could not account for the associations we observed in this study. Confounding by other factors is always a concern in epidemiologic studies. We observed little confounding when we carefully adjusted for known risk factors. Although it is possible residual confounding may still exist, for example from dietary factors not considered in this study, additional factors are not likely to explain the strength of the observed associations. Other strengths of our study include the population-based design, the estimation of folate intake in a population of nonusers of alcohol and vitamin supplements, and the large sample size that facilitated examination of modifying effects.

In summary, we found that, although there was no overall relationship between *MTHFR* genotype and breast cancer risk, women with low intake of folate and who are homozygous for the *MTHFR* 677 T polymorphism may be at substantially increased risk for breast cancer. Our data also suggest this association may be further modified by vitamin B12, vitamin B6, and methionine intake. This study adds support to the literature that one-carbon metabolism and *MTHFR* polymorphisms have a role in carcinogenesis and may be important in breast carcinogenesis. The results of this study potentially have important implications for population public health measures such as food fortification.

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Table I. Comparison of cases and controls by selected descriptive characteristics, Shanghai Breast Cancer Study, 1996-1998.

Subject Characteristic	Cases (n=1144)	Controls (n=1236)	P-value ^a
Age (years), mean \pm SD	46.4 \pm 9.9	46.7 \pm 8.8	0.42
Education, %			
No formal education	3.8	6.0	
Elementary school	8.5	8.6	
Middle or high school	75.8	75.3	
College or above	12.0	10.1	<0.05
Breast cancer in first-degree relative, %	3.4	2.4	0.15
Ever had breast fibroadenoma, %	9.7	5.2	<0.01
Age at menarche (years)	14.5 \pm 1.6	14.7 \pm 1.7	<0.01
Ever had a live birth, %	94.9	95.9	0.27
Number of live births, mean \pm SD	1.5 \pm 0.8	1.5 \pm 0.9	0.19
Age at first live birth (years), mean \pm SD	26.8 \pm 4.1	26.2 \pm 3.8	<0.01
Post-menopausal, %	33.3	36.3	0.13
Age at menopause (years), mean \pm SD	48.2 \pm 4.6	47.4 \pm 5.0	0.03
Physically active past 10 years, %	19.3	25.8	<0.01
BMI (kg/m ²), mean \pm SD	23.6 \pm 3.4	23.2 \pm 3.4	0.02
Waist-to-hip ratio, mean \pm SD	0.81 \pm 0.06	0.80 \pm 0.06	<0.01
Daily animal food intake (g), mean \pm SD	90.4 \pm 61.8	79.4 \pm 50.1	<0.01
Daily plant food intake (g), mean \pm SD	501 \pm 275	496 \pm 278	0.73
Daily folate intake (μ g), mean \pm SD	287 \pm 141	303 \pm 179	0.02
Daily methionine intake (g), mean \pm SD	1.72 \pm 0.60	1.65 \pm 0.56	<0.01
Daily vitamin B12 intake (μ g), mean \pm SD	4.77 \pm 4.11	4.69 \pm 4.20	0.66
Daily vitamin B6 intake (mg), mean \pm SD	1.83 \pm 0.60	1.77 \pm 0.57	0.03
Daily energy intake (kcal), mean \pm SD	1875 \pm 467	1852 \pm 459	0.23

^a For χ^2 test (categorical variables) or *t* test (continuous variables).

Table II. *MTHFR* genotype frequencies and adjusted ORs for breast cancer among Chinese women, Shanghai Breast Cancer Study, 1996-1998.

<i>Genotype</i> ^a	Cases n (%)	Controls n (%)	Age-adjusted OR (95% CI)	Multi-Adjusted OR (95% CI) ^b
<u>C677T</u>				
CC	374 (33.6)	387 (33.4)	1.00 (ref)	1.00 (ref)
CT	555 (49.9)	577 (49.7)	1.00 (0.83-1.20)	1.01 (0.84-1.22)
TT	183 (16.5)	196 (16.9)	0.97 (0.76-1.24)	0.98 (0.77-1.27)
<u>A1298C</u>				
AA	768 (68.5)	824 (68.2)	1.00 (ref)	1.00 (ref)
AC	311 (27.7)	344 (28.5)	0.97 (0.81-1.16)	0.96 (0.80-1.15)
CC	42 (3.8)	40 (3.3)	1.13 (0.72-1.75)	1.15 (0.74-1.81)
<u>Combined</u>				
<u>A1298C-AA</u>				
C677T-CC	196 (18.0)	180 (15.9)	1.00 (ref)	1.00 (ref)
C677T-CT	375 (34.4)	410 (36.2)	0.84 (0.66-1.07)	0.86 (0.67-1.10)
C677T-TT	179 (16.4)	184 (16.3)	0.89 (0.67-1.19)	0.91 (0.68-1.22)
<u>A1298C-AC/CC</u>				
C677T-CC	171 (15.7)	203 (17.9)	0.77 (0.58-1.03)	0.77 (0.58-1.03)
C677T-CT/TT	168 (15.4)	155 (13.7)	0.99 (0.74-1.34)	1.01 (0.75-1.37)

^a The frequencies of the 677T allele were 41.4% in cases and 41.8% in controls ($p=0.81$) and the frequencies of the 1298C allele were 17.6% in cases and 17.5% in controls ($p=0.95$).

^b All ORs are adjusted for age, personal history of fibroadenoma, waist-to-hip ratio, age at first live birth, physical activity, menopausal status, and total meat intake.

Table III. Association of *MTHFR* genotypes with breast cancer, stratified by age and menopausal status among Chinese women, the Shanghai Breast Cancer Study, 1996-1998.

Genotype	Age				Menopausal Status			
	<45		≥ 45		Pre		Post	
	Cases/ controls	Adjusted OR (95% CI) ^a	Cases/ controls	Adjusted OR (95% CI) ^a	Cases/ controls	Adjusted OR (95% CI) ^a	Cases/ controls	Adjusted OR (95% CI) ^a
<u>C677T</u>								
CC	157/165	1.00 (ref)	217/222	1.00 (ref)	258/252	1.00 (ref)	116/135	1.00 (ref)
CT	236/242	1.05 (0.79-1.40)	319/335	0.99 (0.77-1.27)	371/373	0.99 (0.79-1.25)	184/204	1.07 (0.77-1.48)
TT	69/72	1.00 (0.67-1.50)	114/124	0.95 (0.68-1.31)	114/119	0.95 (0.69-1.30)	69/77	1.07 (0.70-1.63)
<u>A1298C</u>								
AA	312/327	1.00 (ref)	456/497	1.00 (ref)	506/506	1.00 (ref)	262/318	1.00 (ref)
AC	126/157	0.80 (0.60-1.07)	185/187	1.10 (0.86-1.41)	206/242	0.85 (0.68-1.06)	105/102	1.25 (0.91-1.71)
CC	22/16	1.54 (0.79-3.00)	20/24	0.92 (0.50-1.72)	33/24	1.37 (0.80-2.36)	9/16	0.70 (0.30-1.60)
Combined								
<u>A1298C-AA</u>								
C677T-CC	72/77	1.00 (ref)	124/103	1.00 (ref)	126/116	1.00 (ref)	70/64	1.00 (ref)
C677T-CT	166/162	1.12 (0.75-1.66)	209/248	0.71 (0.51-0.98)	254/249	0.95 (0.70-1.30)	121/161	0.73 (0.48-1.11)
C677T-TT	69/66	1.09 (0.68-1.75)	110/118	0.79 (0.54-1.15)	112/112	0.93 (0.64-1.34)	67/72	0.92 (0.56-1.49)
<u>A1298C-AC/CC</u>								
C677T-CC	80/87	0.95 (0.60-1.48)	91/116	0.66 (0.45-0.97)	126/135	0.83 (0.58-1.19)	45/68	0.67 (0.40-1.13)
C677T-CT/TT	63/76	0.85 (0.53-1.37)	105/79	1.18 (0.79-1.77)	107/116	0.83 (0.58-1.21)	61/39	1.62 (0.94-2.78)

^a All ORs are adjusted for age, personal history of fibroadenoma, waist-to-hip ratio, age at first live birth, physical activity, and total meat intake.

Table IV. Stratified analyses of the association of *MTHFR* genotype and folate intake with breast cancer risk among Chinese women, Shanghai Breast Cancer Study 1996-1998.

Genotype	Daily Folate Intake ^a								P for trend
	Q4 (High)		Q3		Q2		Q1		
	Cases/ controls	Adjusted OR (95% CI) ^a	Cases/ controls	Adjusted OR (95% CI) ^a	Cases/ controls	Adjusted OR (95% CI) ^a	Cases/ controls	Adjusted OR (95% CI) ^a	
Stratified by Genotype									
C677T									
CC	69/88	1.00 (ref)	96/86	2.06 (1.25-3.41)	90/91	2.13 (1.20-3.80)	81/86	2.51 (1.29-4.89)	0.02
CT	103/117	1.00 (ref)	135/142	1.21 (0.81-1.81)	133/137	1.29 (0.82-2.03)	145/136	1.65 (0.99-2.75)	0.06
TT	29/53	1.00 (ref)	47/44	2.70 (1.31-5.57)	49/39	3.82 (1.69-8.66)	47/38	4.75 (1.82-12.4)	0.003
	P for interaction =0.048								
A1298C									
AA	140/192	1.00 (ref)	184/192	1.59 (1.13-2.22)	195/185	1.90 (1.30-2.76)	194/186	2.23 (1.44-3.45)	0.0006
AC/CC	63/81	1.00 (ref)	92/84	1.71 (1.02-2.87)	79/91	1.52 (0.84-2.77)	85/92	1.85 (0.95-3.63)	0.18
	P for interaction =0.71								
A1298C-AA									
C677T-CC	42/45	1.00 (ref)	43/43	1.51 (0.75-3.03)	48/38	2.12 (0.96-4.12)	43/40	2.30 (0.89-5.95)	0.07
C677T-CT	67/81	1.00 (ref)	94/101	1.30 (0.80-2.13)	90/98	1.35 (0.78-2.32)	100/98	1.78 (0.96-3.30)	0.08
C677T-TT	29/52	1.00 (ref)	45/41	2.89 (1.38-6.06)	48/36	4.20 (1.83-9.66)	46/36	5.31 (1.99-14.2)	0.002
	P for interaction =0.06								
Stratified by Folate Intake									
C677T									
CC	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	
CT	1.23 (0.79-1.90)	0.86 (0.58-1.26)	0.86 (0.58-1.26)	0.96 (0.65-1.40)	0.96 (0.65-1.40)	1.12 (0.76-1.65)	1.12 (0.76-1.65)	1.12 (0.76-1.65)	
TT	0.73 (0.41-1.31)	0.95 (0.57-1.59)	0.95 (0.57-1.59)	1.27 (0.76-2.14)	1.27 (0.76-2.14)	1.33 (0.78-2.28)	1.33 (0.78-2.28)	1.33 (0.78-2.28)	
P for trend	0.51	0.71	0.71	0.49	0.49	0.31	0.31	0.31	
A1298C									
AA	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	
AC/CC	1.01 (0.67-1.53)	1.10 (0.76-1.59)	1.10 (0.76-1.59)	0.88 (0.60-1.27)	0.88 (0.60-1.27)	0.87 (0.60-1.26)	0.87 (0.60-1.26)	0.87 (0.60-1.26)	
A1298C-AA									
C677T-CC	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)	
C677T-CT	0.86 (0.49-1.53)	0.92 (0.55-1.54)	0.92 (0.55-1.54)	0.72 (0.43-1.21)	0.72 (0.43-1.21)	0.96 (0.56-1.61)	0.96 (0.56-1.61)	0.96 (0.56-1.61)	
C677T-TT	0.58 (0.30-1.12)	1.07 (0.58-1.97)	1.07 (0.58-1.97)	1.05 (0.57-1.95)	1.05 (0.57-1.95)	1.21 (0.65-2.27)	1.21 (0.65-2.27)	1.21 (0.65-2.27)	
P for trend	0.13	0.63	0.63	0.81	0.81	0.70	0.70	0.70	

^a All ORs are adjusted for age, personal history of fibroadenoma, waist-to-hip ratio, age at first live birth, physical activity, and total energy, meat, vitamin B12, vitamin B6, and methionine intakes.

Table V. Association of folate with breast cancer risk stratified by *MTHFR* C677T genotype and cofactor intake among Chinese women, Shanghai Breast Cancer Study 1996-1998.

Folate Cofactor Intake	Folate Intake OR (95% CI) ^a			P for trend	P for interaction
	T3 (High)	T2	T1		
Vitamin B12					
Low					
CC	1.00 (ref)	1.75 (0.68-4.51)	2.08 (0.66-6.54)	0.25	0.56
CT	1.00 (ref)	0.87 (0.42-1.77)	1.06 (0.47-2.38)	0.79	
TT	1.00 (ref)	0.92 (0.23-3.67)	2.65 (0.53-13.27)	0.11	
Medium					
CC	1.00 (ref)	1.77 (0.82-3.80)	2.19 (0.83-5.79)	0.11	0.45
CT	1.00 (ref)	1.53 (0.83-2.79)	1.65 (0.77-3.53)	0.20	
TT	1.00 (ref)	2.75 (0.84-9.04)	5.89 (1.25-27.81)	0.03	
High					
CC	1.00 (ref)	1.29 (0.60-2.73)	1.53 (0.57-4.06)	0.39	0.02
CT	1.00 (ref)	0.98 (0.53-1.80)	1.06 (0.51-2.21)	0.88	
TT	1.00 (ref)	4.44 (1.52-12.96)	2.39 (0.64-8.90)	0.12	
P for 3-way interaction = 0.47					
Vitamin B6					
Low					
CC	1.00 (ref)	0.74 (0.14-3.97)	1.13 (0.21-5.98)	0.30	0.70
CT	1.00 (ref)	1.42 (0.33-6.21)	1.82 (0.43-7.72)	0.26	
TT	1.00 (ref)	0.58 (0.02-15.58)	1.91 (0.07-48.90)	0.06	
Medium					
CC	1.00 (ref)	1.53 (0.70-3.33)	1.76 (0.71-4.37)	0.24	0.70
CT	1.00 (ref)	1.44 (0.80-2.61)	1.26 (0.64-2.48)	0.57	
TT	1.00 (ref)	3.12 (0.98-9.96)	5.38 (1.42-20.33)	0.02	
High					
CC	1.00 (ref)	2.25 (1.15-4.42)	1.61 (0.32-8.00)	0.04	0.13
CT	1.00 (ref)	0.92 (0.55-1.54)	0.57 (0.17-1.86)	0.44	
TT	1.00 (ref)	2.81 (1.07-7.41)	0.63 (0.08-4.68)	0.23	
P for 3-way interaction = 0.24					
Methionine					
Low					
CC	1.00 (ref)	1.89 (0.42-8.60)	1.52 (0.31-7.45)	0.88	0.98
CT	1.00 (ref)	0.78 (0.32-1.89)	0.88 (0.34-2.28)	0.98	
TT	1.00 (ref)	0.46 (0.08-2.68)	1.06 (0.16-7.07)	0.41	
Medium					
CC	1.00 (ref)	1.29 (0.61-2.74)	2.21 (0.86-5.71)	0.09	0.91
CT	1.00 (ref)	1.25 (0.66-2.38)	1.50 (0.70-3.22)	0.30	
TT	1.00 (ref)	2.13 (0.69-6.56)	3.44 (0.83-14.27)	0.09	
High					
CC	1.00 (ref)	1.79 (0.88-3.64)	1.70 (0.50-5.81)	0.16	0.19
CT	1.00 (ref)	1.03 (0.60-1.76)	1.11 (0.48-2.54)	0.82	
TT	1.00 (ref)	4.05 (1.46-11.18)	3.43 (0.86-13.75)	0.03	
P for 3-way interaction = 0.89					

^a All ORs are adjusted for age, personal history of fibroadenoma, waist-to-hip ratio, age at first live birth, physical activity, menopausal status, and total energy, meat, and intake of the other two cofactors